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(54) Title: FLUORESCENT DYES FOR IDENTIFICATION AND ENUMERATION OF VIABLE CELLS IN MILK

(57) Abstract

An improved process is provided for the detection, identification, and/or enumeration of viable cells in bovine milk wherein viable cells are selectively labeled with a fluorescent dye and then identified and/or counted. In this process, esterase-dependent dyes, nucleic acid binding dyes, and dyes that detect intracellular oxidative activity are used.

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FLUORESCENT DYES FOR IDENTIFICATION AND ENUMERATION OF VIABLE CELLS IN MILK

Field of the Invention

The present invention relates in general to the identification and enumeration of viable cells in milk and, more particularly, to the use of fluorescent dyes to label viable cells in bovine milk for identification.

Background of the Invention

Bovine mastitis, or inflammation of the udder, causes significant economic losses to the dairy industry amounting to approximately $2 billion per year in the United States. These costs are caused by those directly associated with treatment and by short and long term reductions in milk quantity and/or quality. These losses could be mitigated by improved diagnostic procedures that were sufficiently sensitive to detect preclinical mastitis and that were capable of providing more accurate prognostic information during treatment.

Although mastitis is characterized by the appearance in the milk of inflammatory cells, i.e., polymorphonuclear and mononuclear leukocytes, mastitis is most commonly diagnosed by measuring the "somatic" cell count. The somatic cell count includes both the inflammatory cells and other cell types such as epithelial cells that are shed from the udder and that may occur in milk from either normal or mastitic cattle. High somatic cell counts are considered indicative of

mastitis and there are legal limits on the somatic cell count permitted in milk for human consumption. However, cattle with low somatic cell counts consisting primarily of granulocytes may have mastitis that cannot be detected by this test. Conversely, cattle can have high somatic cell counts caused by large numbers of shed epithelial cells or cell fragments, particularly late in lactation. Since the somatic cell count fails to distinguish between live and dead cells or between cell types, these cattle may be incorrectly perceived to have mastitis. Thus, the somatic cell count can produce both false negative and false positive results. However, the somatic cell count can be automated, does not require highly trained technicians, and is not especially labor-intensive. Therefore, the somatic cell count is relatively inexpensive ($0.20-0.30 per test) and can be performed rapidly (180-220 samples per hour) making it capable of being used to screen large numbers of cattle on a regular basis.

An improved mastitis test would measure the inflammatory cells that are actually characteristic of mastitis. Of the inflammatory cells, the granulocytes are the most significant in diagnosing mastitis. Granulocytes are the first inflammatory cells present in mastitic milk and their continued presence indicates that the problem causing the mastitis has not been solved. Granulocytes may also be accompanied by other leukocytes including lymphocytes and monocyte/macrophage type cells. Thus, an improved test would detect these types of cells and allow one to distinguish these cells from cells or cell fragments that can occur in normal milk. An improved test would also need to be inexpensive and capable of analyzing large numbers of samples. These requirements preclude manual techniques based on the preparation and microscopic examination of cell smears from milk. It is, therefore, desirable to have a rapid

procedure to enumerate and/or identify viable inflammatory cells. Such a test could be used to screen dairy cattle for pre-clinical mastitis and to monitor milk during treatment for mastitis in order to determine the effectiveness of therapy.

The Prior Art

In the United States, the standard procedure for determining the somatic cell count of milk uses a Foss instrument. This instrument can process over 200 samples per hour rendering it suitable to screen the large-numbers of samples associated with routine screening of dairy cattle. In operation, the instrument withdraws a measured quantity of milk, dilutes it in a detergent-ethidium bromide solution to lyse all cells and label the cell nuclei, deposits a volume of labeled material onto a rotating wheel, and then counts the number of fluorescent spots corresponding to cell nuclei. Since all the dilution factors are known, these counts of nuclei can be converted to the cell concentration in the original milk sample. The detergent treatment lyses cells to render the nuclei available to the ethidium bromide dye and also breaks up butterfat globules that can clog some types of instruments. However, the detergent lysis also prevents the instrument from being able to distinguish different types of cells or to distinguish viable from dead cells. As a result, early cases of mastitis in which the milk contains a small number of inflammatory cells can go undetected. On the other hand, milk containing large numbers of normal epithelial cells but no inflammatory cells would be incorrectly identified as "mastitic". Similarly, the instrument cannot detect a change in the types of cells present in milk, such as can occur during successful treatment of mastitis. These shortcomings mean that the somatic cell count as currently performed with this type of instrumentation cannot meet the ideal objectives

listed above. An alternative type of instrument, namely a flow cytometer, could enable one to distinguish various cell types and to distinguish viable from dead cells. The following paragraphs will summarize why flow cytometry has not supplanted the currently established technique.

Flow cytometry, i.e., the simultaneous measurement of one or more parameters for each individual cell in a single cell suspension, has been in use for over 20 years. One type of single parameter flow cytometer, the electronic particle counter as exemplified by the "Coulter Counter", has been in widespread use in clinical laboratories for over 20 years. These instruments count "cells" by measuring an alteration in electric current flow caused by a particle as it passes between two electrodes. The magnitude of the alteration is related to particle size so that cells or particles of different sizes can be distinguished and enumerated. Although widely used to enumerate cells in blood, these types of instruments could not easily be used to enumerate cells in milk because of the presence of butterfat globules that can clog the instrument and/or "confuse" the instrument and be detected as "cells". Cells could be separated from the butterfat globules by centrifugation, but the additional steps and the problems associated with distinguishing cells from other particles have essentially precluded interest in developing electronic particle counters to analyze cells in milk. Therefore, the simplest type of flow cytometry has been deemed unsuitable for analyzing cells in milk.

More complex flow cytometers capable of measuring several parameters simultaneously were developed about 20 years ago. In addition to measuring electronic cell volume, these instruments could also measure light scatter and/or fluorescence emissions from appropriately labeled cells. In the mid-1970s, Ortho, one of the early manufacturers of flow cytometers, developed and published

a Protocol to enumerate and/or differentiate cells in milk by labeling with the nucleic acid binding dye acridine orange. This procedure allowed one to distinguish cells from butterfat globules and to identify different types of cells in milk. Nevertheless, the procedure did not gain commercial acceptance for several reasons. First, the instrument for which the procedure was developed cost upwards of $200,000 (in mid-1970s dollars). Second, the instrument was expensive to maintain, and required an expensive computer and expensive peripherals, such as printers and mass storage devices. Third, the instrument required a skilled operator and the data that were produced required complex interpretation. Fourth, and perhaps most important, the instrument was incapable of processing samples much faster than one per 1-3 minutes or only 160-500 samples per eight hour day. This rate is about an order of magnitude slower than would be useful in a commercial laboratory, which might need to process thousands of samples per day. Although flow cytometers and computers have become much less expensive in the last 15 years (less than $100,000 in current dollars), the other limitations still pertain. Thus, there has been little or no development of flow cytometers for commercial analysis of milk in the United States.

In addition to virtually no commercial interest in flow cytometric analysis of milk, there has also been little research application of flow cytometry to the analysis of milk or to other veterinary areas. In the late 1970's, monoclonal antibodies that identify cell surface differentiation antigens were developed. Combined with flow cytometry, these antibodies to human cells have been used in literally thousands of published reports. New, relatively inexpensive flow cytometers are now being sold to clinical laboratories at the rate of hundreds per year. New types of fluorescent dyes are being developed and used to measure a variety of cellular

parameters, e.g., the content of nucleic acid to analyze cancer cells, the intracellular pH or the intracellular concentration of other ions such as Ca^{2+} that change during cell functions, the intracellular oxidative activity, etc. Nevertheless, the application of these powerful techniques to veterinary problems in general and the analysis of milk in particular has lagged years behind.

There have been several reasons for the failure to develop and apply these techniques to veterinary problems. First, the perception that there were few commercial applications has diminished or eliminated most R&D support from commercial sources. Since there are no manufacturers of flow cytometers in the United States that are targeting the veterinary market, these companies are also not putting resources into developing applications. Second, research grant support for these types of studies has also been severely limited. The granting agencies with the largest budgets, namely, the National Institutes of Health and the National Science Foundation, have not supported veterinary work to any significant extent. The United States Department of Agriculture also provides research funding, but its total research budget is very small. Furthermore, the USDA appears to have the attitude that flow cytometry will never have significant application to veterinary studies. This type of thinking is exemplified by the following excerpt from the 1985 textbook. Veterinary Immunology, Academic Press, New York, p 41 (1985).

> The costliness of the monoclonal antibodies and of the instrument [flow cytometer] has limited the use of this machine, with lymphocytes from domestic animals, to a few research institutes. At the moment it is difficult to visualize such machines being used outside these institutes for clinical studies on animals.

Third, in university settings where flow cytometers and veterinary scientists may both be present, there have been relatively few interactions between them. The analytical cytologists have not appreciated the possible applications to veterinary areas and the veterinary scientists have not realized that flow cytometry could help them. Thus, a circular feedback loop has been created that has greatly reduced the development of flow cytometric applications for veterinary areas.

The end result of all these factors is illustrated by the extremely small number of published studies using flow cytometry to analyze cells in milk. About 10 years after the Ortho Protocol was developed, Saad and Hageltorn, Acta Vet.Scand. 26:289-307, 1985, used flow cytometric procedures to measure the phagocytic activity of the inflammatory cells in milk. Later, this same group used the acridine orange labeling technique to examine the properties of the leukocytes in the bovine milk and blood. Hagelhorn M, and Saad MA, Am.J. Vet. Res. 47:2012-2116, 1986.

The inventor's laboratory developed and published a new flow cytometric technique to analyze cells in milk in 1988. Redelman, D., et al., Cytometry 9:463-468, 1988. This procedure simultaneously measured cell volume, right angle light scatter, and the intracellular fluorescence produced in live cells by the de-esterification of carboxymethylfluorescein diacetate (CMFDA). These properties distinguished live and dead cells and could be used to identify the cell types present in milk. However, there was substantial fluorescence produced in some dead or damaged cells, particularly in the disintegrating granulocytes in mastitic milk The

fluorescence intensity of intact granulocytes was less than ten-fold greater than that of disintegrating granulocytes and/or other dead cells in milk.

In 1989, Salgar et al., FASEB Journal 3:A320 Abstract #546, 1989, used flow cytometry to measure the oxidative activity of granulocytes isolated from milk. These tests measured the intracellular fluorescence produced in granulocytes by de-esterification and subsequent oxidation of dichlorodihydrofluorescein diacetate (DCHF-DA) to dichlorofluorescein (DCF). These investigators concluded that the granulocytes in mastitic milk were deficient in their ability to produce an oxidative response.

In 1990, the inventor's company reported additional flow cytometric studies of cells in milk at the most recent International Meeting of the Society for Analytical Cytology. Redelman D, Cytometry Supplement 4:41 Abstract #273, 1990. These experiments used the DCF procedure to measure the intracellular oxidative activity of granulocytes from milk before and after stimulation. In addition, these cells were also labeled with the membrane potential sensitive dye 3',3'-dihexyloxacarbocyanine iodide $DiOC_6(3)$. Shapiro HM, et al., Proc. Nat. Acad. Sci. USA 76:5728, 1979. The results demonstrated that granulocytes in mastitic milk are partially "activated", i.e., the activities of several intracellular enzymes are increased to improve the phagocytic and bacteriolytic functions of granulocytes. However, in contrast to the previous report cited above, the pre-activated granulocytes in mastitic milk clearly retained the ability to respond to stimuli.

At the same meeting, Williams, et al., Cytometry Supplement 4:108 Abstract #629A, 1990, reported flow cytometric analyses of the phagocytic activity of cells in milk. This meeting was attended by nearly 900 individuals, including a large proportion of the

analytical cytologists from all over the world, and there were only these two reports of analyses of cells in milk. Since there have been very few individuals or laboratories using flow cytometry to examine cells in milk, the pace of development has been extremely slow. New types of dyes or new analytical procedures developed in other systems have not been modified for use in the analyses of cells in milk.

Flow cytometric studies of cells in milk have demonstrated that selective detection of viable cells in milk would improve the sensitivity of detecting mastitis and would improve the accuracy of prognosis in cows being treated for mastitis. However, flow cytometric techniques would have to be modified in order to have commercial application because the major problem with flow cytometric analysis is that existing instruments do not have sufficient rates of sample analysis to process the large number of milk samples that would be required for regular routine screening tests. Options for doing so include developing faster flow cytometers or developing other systems to measure viable cells. However, faster flow cytometers would have the disadvantage of being costly. In addition, many of the other perceived disadvantages of flow cytometry would remain. Thus, developing faster flow cytometers does not appear the most likely to succeed. Alternatively, other systems could be devised that would still permit identifying viable cells in milk.

The approach selected in accordance with this invention is to label the cells in milk with a dye that selectively is fluorescent in viable cells. A dye sufficiently selective for viable cells would allow measuring the fluorescence after depositing the cells on a filter, concentrating them by centrifugation, or other techniques that permit measuring the fluorescent signal associated with the cells. The dyes that have been used in the previously reported flow cytometric tests to

identify cells in milk, namely acridine orange and CMFDA, are not suitable for these types of tests. In the case of acridine orange, it labels nucleic acid in cell fragments and would not distinguish viable from dead cells. CMFDA, although generally considered a "vital" dye that selectively labels only viable cells, was found to produce substantial fluorescence in dead cells. Therefore, since neither of the previously reported flow cytometric procedures for identifying viable cells in milk appeared to be suitable, other types of dyes and labeling procedures that have not previously been applied to cells in milk were considered.

Flow cytometry was used to evaluate fluorescent dyes for their suitability. The most important criteria are 1) how effectively these different dyes discriminate between viable and dead or damaged cells, and 2) the potential of these dyes for discriminating among different types of cells. Flow cytometry permits precise quantitive comparisons. For example, since dead or damaged cells can outnumber viable inflammatory cells in milk by as much as 10:1, it is important to identify dyes that produce at least a 10:1 difference in labeling intensity between viable and dead cells. Flow cytometric measurements permit making these comparisons. Several of the variables that could be encountered in collecting and analyzing milk were also examined including determining the differences between milk samples collected at the beginning of milking vs. those collected at the end of milking, the effects of allowing milk samples to stand for various times before labeling, and the effect of allowing labeled cells to stand for various times before analysis.

OBJECTS OF THE INVENTION

It is an object of the invention to provide an improved process for rapidly and accurately detecting, identifying, and/or enumerating viable cells in bovine milk to determine milk quality, to identify cattle with pre-clinical mastitis, and to monitor the cells in milk from mastitic cattle during treatment.

SUMMARY OF THE INVENTION

This invention relates to a process for detecting, identifying, and/or enumerating viable cells in bovine milk wherein viable cells are labeled with a fluorescent dye and then identified and/or counted, wherein the viable cells are selectively labeled by adding to the milk:

(a) at a temperature at which intracellular esterase enzymes are active, an esterified precursor of a fluorescent dye that is de-esterified in the viable cells producing fluorescent intensities at least ten-fold greater than in dead or damaged cells; or

(b) at a temperature at which appropriate intracellular enzymes are active and at which nucleic acid is intact, a precursor of a nucleic-acid binding dye that is converted to the fluorescent nucleic acid binding form by the intracellular enzymes and which then binds to the nucleic acid of intact cells; or

(c) at a temperature at which intracellular oxidative processes are active, a fluorescent dye precursor which can penetrate the membrane of an intact cell and which is converted by intracellular oxidation to a charged fluorescent dye.

DETAILED DESCRIPTION

The objects of this invention, viz., to identify fluorescent dyes which can distinguish cells in milk with a sufficiently high signal to noise ratio for use in screening tests and determine the conditions required to

permit them to do so, have now been met. A variety of different types of dyes have been evaluated. Dyes from several categories have been found to be appropriate whereas, unexpectedly, many others have not.

The fluorescent dyes and dye precursors which can selectively label viable cells in milk can be divided into the four categories listed below, based upon the manner in which they selectively identify intact cells. The dyes can be used to label cells from milk by adding the dye directly to a sample of the milk or to a suspension of the cells separated from milk. Dyes from all four categories can be used in flow cytometric studies to enumerate and/or identify cells in milk. However, only the dyes in the first three categories can be used in accordance with this invention because they have sufficient discriminatory capacity to be used with other detection systems suitable for analyzing large numbers of samples.

1) Esterified esterase-dependent dyes.

Charged fluorescent molecule do not freely cross the membranes of intact cells. By esterification, some of these molecules can be converted to uncharged and non-fluorescent derivatives that readily diffuse into cells. In viable cells containing the appropriate esterases, they are de-esterified back to charged fluorescent molecules that are trapped inside the intact cells. This approach has been used to make esterase-dependent "vital" dyes and to load the precursors of other types of fluorescent molecules. The one dye of this type that has been used with cells in milk, namely CMFDA, discriminated relatively poorly between viable and dead cells. The reasons for this poor performance were not obvious since there are at least four factors that can affect the labeling with these dyes. These factors are 1) the rate at which the

esterified precursor crosses membranes of intact cells, 2) the rate at which intracellular esterases hydrolyze the precursor molecules, 3) the rate at which the charged fluorescent dye "leaks", and 4) the fluorescence quantum yield of the dye after cleavage by the intracellular esterases. A series of esterase sensitive dyes was examined in order do determine the importance of these different factors.

One of the major problems in using these dyes in many systems is "leakage" of the dye out of intact cells after they have been labeled. To counter the leakage problem, derivatives with a greater charge have been prepared. For example, 5(and-6)carboxy-4',5'-dimethylfluorescein diacetate (CMFDA), the related compound 5(and-6)-carboxyfluorescein diacetate (CFDA), and 5(and-6)-sulfofluorescein diacetate (SFDA) have all been developed as superior vital dyes that are well-retained. In comparison with CMFDA, CFDA and SFDA were actually much poorer in discriminating between live inflammatory cells and dead cells in milk. Thus, these esterase-dependent vital dyes are not suitable for use in this invention. In contrast to these dyes, fluorescein, the fluorescent hydrolysis product of fluorescein diacetate (FDA), has less charge and is known to leak at a faster rate. However, FDA labeled cells very intensely and produced 30-300 fold higher intensity in viable cells than in dead cells. Thus, FDA is one esterase-dependent vital dye that is satisfactory. Although FDA was one of the prototypical esterase-dependent vital dyes, it has not been tested in this application previously.

The more highly charged derivatives, such as CFDA or SFDA, are known to be poorer substrates of intracellular esterases in addition to being trapped more effectively. Since these dyes had to used at higher concentrations in order to label cells, it suggested the substrate effectiveness could be an important consideration. One way in which this problem has been approached is to use

acetoxymethyl (AM) esters instead of acetate esters. For example, 2',7'-(bis)(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) has been used to measure intracellular pH and as a dye to measure cytotoxicity reactions. BCECF-AM was tested and found to label cells with efficiency similar to that of FDA. A second AM esterified dye, namely the Ca^{2+} indicator dye Indo-1 AM, also labels cells with high efficiency. Although Indo-1 AM has not been as thoroughly tested, it appears it may also be useful in analyzing cells in milk. Furthermore, it suggests that fluorescent dyes esterified with the acetoxymethyl group may be generally useful in analyzing cells in milk.

Esterified derivatives of fluorescent dyes that are able to enter cells and be de-esterified in situ to a charged fluorescent form thus are a category of dyes that includes individual dyes which can be used in this invention. For a specific dye in this category to be useful in this invention, it must be able to label viable inflammatory cells in milk at least 10-fold more intensely than dead cells or other cell types in milk. The suitability of any specific member of this category can be determined by measuring the fluorescence it produces in an aliquot of leukocytes isolated from blood that is suspended in buffered saline and allowed to stand at room temperature for sufficient time to cause cell death in a portion of the cells in the suspension. Two esterase-dependent dyes, namely FDA and BCECF-AM, meet these criteria. Preliminary tests indicate that the calcium-sensitive dye Indo-1 AM is also satisfactory. However, a number of widely used esterase-dependent vital dyes, including CFDA, CMFDA, and SFDA, have been shown to be unsatisfactory because they fail to discriminate adequately between viable inflammatory cells and other cells in milk.

2. Nucleic acid binding dyes.

In terms of the ability to label live and/or dead cells, fluorescent dyes that bind to and label nucleic acids can be divided into three categories:

a) Dyes that do not effectively cross intact cell membranes. Dyes of this type, as typified by ethidium bromide and propidium iodide, are usually charged molecules. When added to cell suspension containing intact viable cells, dead cells with damaged membranes, and fragments of cells and cell nuclei, these dyes selectively label the dead cells and the cell fragments. Therefore, nucleic acid binding fluorescent dyes of this type are not suitable for use in the process of this invention.

b) Dyes that are able to enter live cells and fluorescently label nucleic acids without further chemical conversion. This category is typified by dyes such as Hoechst 33342 and pyronin-Y that can be taken-up by viable cells and label DNA and/or RNA. However, these dyes also label dead cells and nuclear fragments so they also are unsuitable for use in this invention.

c) Fluorescent nucleic acid binding dyes that have been chemically modified such that 1) their fluorescence properties and/or nucleic acid binding properties have been modified, 2) they are able to enter viable cells, and 3) they are able to be converted back to the fluorescent nucleic acid binding dye by chemical reaction inside viable cells. An example of a dye which falls within this category is dihydroethium ("Hydroethidine", HED). HED is a reduced form of ethidium that has altered fluorescence excitation and emission properties and that is able to enter viable cells. Within viable cells, intracellular oxidation converts HED back to ethidium which can then bind to nucleic acid and label it fluorescently. HED has been used to label several types of viable cells selectively, including sperm cells (Ericsson SA et al. Gamete Res.

22:355-368 1989. Assessment of the viability and fertilizing potential of cryopreserved bovine spermatozoa using dual fluorescent staining and two-flow cytometric systems.) but it had not previously been tested with cells in milk. HED has now been tested with cells from milk and demonstrated to label viable cells with high fluorescence intensity, i.e., at least 10,000 fold brighter than the normal autofluorescence level. Furthermore, HED discriminates between viable cells and dead cells at least as effectively as the esterase-dependent dyes FDA or BCECF-AM. Therefore, HED has established that this third category of nucleic acid binding dyes is suitable in this invention. Other nucleic acid binding fluorescent dyes can also be chemically modified to fit the criteria listed above to meet the required labeling criteria and are suitable for this invention.

3. Dyes dependent upon intracellular oxidation.

Most cells contain intracellular oxidative activity that can oxidize dyes such as HED. However, inflammatory cells, particularly granulocytes and macrophages, have considerably higher levels of intracellular oxidative activity. Furthermore, granulocytes that have been activated in an inflammatory reaction in vivo or experimentally in vitro produce an "oxidative burst". One of the standard procedures to measure the oxidative burst by flow cytometry involves the intracellular de-esterification and oxidation of DCHF-DA. This dye has been used in the prior art to measure oxidative responses in granulocytes isolated from milk.

Fluorescent dyes can be chemically reduced such that they have altered fluorescence properties and/or are able to enter viable cells. Inside viable cells, these modified dyes can be oxidized back to the fluorescent form. The resulting fluorescence intensity of the cell is directly related to the activity of the intracellular

oxidative process. Dyes of this type are particularly useful in accordance with this invention to detect inflammatory cells because inflammatory cells, especially granulocytes and macrophages, have elevated levels of intracellular oxidative activity. Furthermore, granulocytes stimulated with agents such as phorbol myristate acetate (PMA) produce as much as 30-fold higher levels of intracellular oxidative activity in a manner of minutes. Thus, cells incubated with these dyes alone or in the presence of PMA will produce different levels of fluorescence depending upon the number of granulocytes in the population.

The prototype dye of this category is dichlorodihydrofluorescein diacetate (DCHF-DA), which is a reduced and esterified nonfluorescent form of dichlorofluorescein (DCF). [NOTE: Although dihydroethidium, which is available under the trademark "Hydroethidine" (HED), is a reduced and uncharged form of ethidium that can enter intact cells and be converted by intracellular oxidation to ethidium ion which can then bind to nucleic acid, it is not included in this category of dyes because the ultimate fluorescence intensity of cells labeled with HED is more dependent upon the nucleic acid content of the cell than upon the content of oxidative activity.] Fluorescent labeling of cells with DCHF-DA requires two steps. First, the esterified dye enters cells where it is de-esterified by intracellular esterases to the charged but nonfluorescent DCHF. Oxidative processes then convert DCHF to the charged fluorescent DCF that remains trapped in the cell. This dye has been used to measure the oxidative response of granulocytes isolated from human blood, Bass DA, et al., J. Immuonol. 130:1910-1917 (1983), from bovine milk, Salgar SK, et al. FASEB Journal 3:A320 Abstract #546, 1989, or from bovine blood or milk. Redelman D, Cytometry Supplement 4:41 Abstract #273, 1990. It has

not been used to screen milk for the presence of inflammatory cells.

Dihydrorhodamine 123, a reduced form of rhodamine 123, is a second example of a dye of this category that has also been used to measure the oxidative response of granulocytes. It differs from DCHF-DA in that dihydrorhodamine 123 only requires intracellular oxidation to convert it to the fluorescent form. It has not yet been used with the cells in milk in any sort of procedure, so it is not known if it produces a sufficient signal to noise ratio to distinguish viable from dead or damaged cells. If it does, then dihydrorhodamine 123 could also be used alone or with PMA to screen for the presence of inflammatory cells in milk.

4. Membrane potential sensitive dyes.

Membrane potential sensitive dyes comprise another category of dyes that have been used to measure viable cells. These dyes are charged fluorescent molecules which accumulate in intact cells because of the electrochemical potential that exists across the cell membranes. Shapiro, et al., Proc. Nat. Acad. Sci. USA 76:5728, 1979. have shown that these dyes could be used in flow cytometric tests to measure membrane potential. These dyes have also formed the basis of a test to identify blood leukocytes. See U.S. 4,343,782.

The fluorescence intensity of cells incubated with membrane potential sensitive dyes is related to the membrane polarization. Mammalian cells have an electrochemical potential across their membranes primarily because of differences in the intra- and extracellular concentrations of Na^+ and K^+ ions. Dead cells or cells with damaged membranes do not have such an electrochemical potential so they do not accumulate these dyes and hence label less intensely. In addition, processes that alter membrane potential by causing

alterations in the content of intracellular ions affect the fluorescence intensity of cells incubated with these dyes. For example, normal granulocytes stimulated with PMA depolarize and lose fluorescence intensity, but not to the level of dead or damaged cells. Since dyes of this sort have been used to detect viable cells isolated from blood, Shapiro HM, U.S. Patent 4,343,782. they were tested with cells from milk in order to determine if they would be suitable.

Cells from milk were tested with 3',3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$ or with Di-4-ANEPPS. The dyes were found to be unsuitable for two reasons. First, the difference in intensity between the intact cells and the damaged cells in milk was relatively small, i.e., less than 3-10 fold. Second, these dyes operate on an equilibrium basis so that any attempts to separate the cells from the dye solution would cause substantial additional loss of fluorescence. Although the membrane potential sensitive dyes were useful in research studies, for example to demonstrate that the granulocytes in milk were pre-activated, (Redelman D, Cytometry Supplement 4:41 Abstract #273, 1990), they are not suitable for use in this invention. Thus, membrane potential sensitive dyes do not comprise one of the categories of dyes included in this invention.

Temperatures used for labeling cells.

The dyes listed above that adequately distinguish viable from dead or damaged cells all require that cells be kept at conditions that do not permanently inactivate the relevant enzymes or disrupt the cell membrane. All of these dyes label the cells at temperatures ranging from "room" temperature, i.e., 22-25° Celsius, to "physiologic" temperature, i.e., 36-39° C. The esterase dependent dyes in the first category label appropriately if the cells are incubated with the dye at room temperature for 10-20 minutes. In order to label the

cells with HED, a dye in the second category that selectively labels nucleic acid in viable cells, it is necessary to incubate the cells with the dye at physiologic temperature for 10-20 minutes. The prototype dye of the third category, namely DCHF-DA, works best if cells are labeled in a two step process. In the first step, the cells are incubated with the dye at room temperature for 10-20 minutes in order to load the cells with de-esterified DCHF. The cells in the dye solution are then transferred to a physiologic temperature either alone or with a stimulating agent such as 100 nm PMA for 10-20 minutes. Labeling with any of these fluorescent dye can be effectively stopped by chilling to 0-4° C. Whatever exact conditions of time and temperature are selected from those listed, it is imperative that these conditions be kept constant. When cells from milk are labeled with constant conditions the resulting fluorescence intensities of different types of cells are highly reproducible.

Procedures for detecting labeled cells.

In order to detect the cells labeled with the fluorescent dyes, the cells must be illuminated with light of the appropriate excitation wavelength. The labeled cells will then fluoresce and emit light at a longer wavelength. The emitted light may be detected qualitatively or quantitatively by using a photomultiplier tube or other light sensing device. These measurements can be made in a flow cytometer which permits measuring individual cells in the milk sample. Alternatively, these measurements can be made after depositing the cells on a filter, after centrifuging them, or after using other conventional techniques to separate the cells from the milk and/or to concentrate the cells into a small volume of liquid or onto a solid surface. The dyes and labeling conditions have been

selected so that it is not necessary to use flow cytometry for analysis.

Other sampling and labeling variables.

The tests that comprise this invention are designed to detect viable cells in milk and/or to distinguish different cell types in milk. Therefore, a number of variables that can affect the number of cells recovered, the types of cells recovered, and/or the viability of the cells recovered were examined. These variables were examined with the established quantitative technique of labeling with CMFDA and measuring the labeled cells by flow cytometry.

In order to compare different methods of milk collection, milk was collected from mastitic cows at the beginning of milking and then again at the end of milking. Milk collected at the start of milking was relatively enriched in granulocytes whereas milk collected at the end of milking was relatively enriched in mononuclear cells. However, all cell types were present in both types of samples. These measurements demonstrate that consistent collection techniques should be used, particularly if one is interested in monitoring mastitic cattle during treatment. Since granulocytes are relatively enriched in samples collected at the beginning of milking, analyzing samples collected in this manner would improve the sensitivity of the tests.

The tests employed in this invention depend on the cells in the milk being viable. Therefore, variables that could affect the viability of the cells in the milk after collection were compared. First, the stability of cells in milk analyzed at various times after collection was determined. Mastitic milk was collected, kept at room temperature and analyzed at various times from approximately 30 minutes to 8 hours after collection, or after standing 20-24 hours after collection. Milk samples analyzed within approximately 8 hours after

collection produced comparable results. Milk samples allowed to stand overnight before analysis had increased numbers of disintegrating granulocytes and comparably reduced numbers of intact granulocytes. Therefore, inflammatory cells are best detected if milk samples were analyzed within 8 hours of collection.

The second variable was the stability of cells after they are labeled. The question of stability was particularly important with cells labeled with FDA since it is known that the intracellular fluorescein "leaks" from viable cells. In order to determine leakage, cells were separated from milk by centrifugation and labeled with either FDA or CMFDA. These cells were then kept at 0-4° C and analyzed by flow cytometry after various times. After 4 hours, there was a 10-38% loss in fluorescence intensity, but no appreciable difference between cells labeled with FDA or with CMFDA. Therefore, cells may be kept at 0-4° C for several hours after labeling before analysis without appreciable loss of fluorescence intensity.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following preferred specific embodiments are, therefore, to be construed as merely illustrative and not limitative of the remainder of the disclosure in any way whatsoever.

In the foregoing and in the following examples, all temperatures are set forth uncorrected in degrees Celsius; and, unless otherwise indicated, all parts and percentages are by weight.

The entire disclosures of all applications, patents and publications, cited above and below, if any, are hereby incorporated by reference.

EXAMPLES

Preparation of Reagents

Cells were suspended in either isotonic buffered saline identical to that used as the sheath fluid in the flow cytometer or in a balanced salt solution (BSS). The isotonic saline sheath fluid (American Scientific Products, McGaw Park, IL 60085, Catalog No. B-3157-12A) contained EDTA and no Ca^{2+} or Mg^{2+}. The BSS contained both Ca^{2+} and Mg^{2+}, each at a concentration of about 1.2mM. The vital dye 5(and -6) carboxy-4',5'-dimethylfluorescein diacetate (CMFDA, Molecular Probes, Eugene, OR 97402, Catalog No. C-367) was solubilized in DMSO at 4.0 mg/ml. The pH-sensitive dye 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxy-methyl ester (BCECF-AM, Calbiochem, La Jolla, CA 92037, Catalog No. 216254) was solubilized in DMSO at 1.0 mg/ml and additionally diluted in DMSO. Fluorescein diacetate (FDA, Sigma Chemical Company, St. Louis, MO 63178, Catalog No. F-5502) was solubilized in DMSO at 4.0 mg/ml and additionally diluted in DMSO. The disodium salt of 5(and-6)-sulfofluorescein diacetate (SFDA, Molecular Probes, Catalog No. S-1129) was solubilized in DMSO at 4.0 mg/ml. Likewise, 5(and-6)-carboxyfluorescein diacetate (CFDA, Molecular Probes, Catalog No. C-195) was also solubilized in DMSO at 1.0 mg/ml. The calcium sensitive dye, Indo-1 acetoxymethyl ester (Molecular Probes, Catalog II, I-1203) was solubilized in DMSO at 1.0mM. Valinomycin (Sigma Chemical, Catalog No. V-0627) was solubilized in DMSO at 1.0 mM. The membrane potential sensitive dye 3',3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$, Molecular Probes, Catalog No. D-273) was solubilized in DMSO at 1.0 mM and additionally diluted in ethanol to produce a 10 μM solution. Another membrane potential sensitive dye, Di-4-ANEPPS (Molecular Probes, Catalog No. D-1129), was solubilized in ethanol at 1.0 or 0.10 mM. The activation of intracellular oxidative activity was measured with 2',7'-dichloro-dihydrofluorescein diacetate (DCHF-DA,

Molecular Probes, Catalog No. D-399) or with 5(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (CDDF-DA, Molecular Probes, Catalog No. C-400). These dyes were solubilized in DMSO at 10 mM. Dihydroethidium "Hydroethidine" (HED), Polysciences, Warrington, PA 18976, Catalog No. 17084 was solubilized in DMSO at 10 mg/ml and additionally diluted in DMSO. The bacterial peptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP, Sigma Chemical, Catalog No. F-3506) was solubilized in DMSO at 100-fold the final desired concentration. Phorbol myristate acetate (PMA, Sigma Chemical, Catalog No. P-8139) was solubilized in ethanol at 100-fold the final intended concentration.

Animals and Preparation of Cells

The animals whose milk was used in most of these tests were the Holstein cattle of the University of Nevada, Reno, dairy herd. In the tests of the membrane potential sensitive dye $DiOC_6(3)$ with whole milk, milk was obtained from mastitic Holstein dairy cattle at the University of California, Davis.

Leukocytes from human and bovine peripheral blood and cells from mastitic bovine milk were examined. Venous blood was collected from normal human donors into heparinized Vacutainer tubes. To prepare leukocytes from human blood, 4-8 ml of blood in a 15 ml conical centrifuge tube was underlain with an equal volume of Histopaque 1077 (Sigma Chemical, Catalog No. 1077-1) and centrifuged for 10-15 minutes at room temperature at 1000 rpm (IEC No. 269 rotor). The cells at the plasma-Histopaque interface, i.e., the peripheral blood mononuclear cells (PBM), were collected, diluted with BSS or with buffered saline sheath fluid, pelleted, and resuspended in a volume approximately equal to the original volume of blood. The layer of Histopaque above the erythrocytes containing the polymorphonuclear neutrophils (PMN) was diluted with BSS or with sheath

fluid, centrifuged, and the washed pellet of cells resuspended in a volume approximately equal to that of the initial blood volume.

Venous blood from Holstein dairy cattle was also collected into heparinized Vacutainer tubes, and leukocytes were prepared by a modification of the procedures used for obtaining human leukocytes. Heparinized bovine blood (2-3 ml) was diluted with an equal volume of sheath fluid, underlain with a volume of Histopaque equal to the volume of the diluted blood, and centrifuged for 20 minutes at 2000 rpm. The PBM were prepared as described above for human cells and resuspended in a volume of BSS or sheath fluid equivalent to the total volume of the original diluted blood. The PMN were obtained by hypotonic lysis of the erythrocyte-PMN pellet. After removing the supernatant layer, the pellet of cells (about 1 ml) was resuspended in sheath fluid to a total volume of 4 ml. The RBCs were then lysed by adding 8 ml of water and mixing. After 30 seconds, 3 ml of 3.40% (w/v) NaCl, i.e., 4x concentrated, was added to restore normal tonicity. The PMN were pelleted and resuspended in BSS or sheath fluid in a volume equivalent to the total volume of the original diluted blood.

Milk samples were collected in several ways for analysis. Composite samples, as collected for Dairy Herd Improvement Association (DHIA) analyses, were obtained for routine herd screening tests. Mid-milk samples from individual quarters were obtained from mastitic cattle. In some cases, pre-milking and post-milking samples from individual quarters of mastitic cattle were compared. In all cases, cells were prepared from milk samples for flow cytometric analysis by centrifuging the milk for 10 minutes at 1000 rpm. The butterfat layer and the whey supernatant layer were removed by vacuum aspiration and the pellet resuspended in a volume of buffered saline sheath fluid or BSS approximately equal to one-half the

original milk volume. In some experiments, cells were prepared from milk at various times after milking in order to determine how long milk could be kept before analysis. Normally, milk samples were processed within 1-3 hours of collection.

Labeling of Cells

Freshly prepared cells from blood or milk, suspended in buffered saline sheath fluid at room temperature, were incubated with 2.0 μl of the stock solution of a dye in DMSO per 1.0 ml of cell suspension for about 15 minutes. The cell suspensions were then chilled and kept on ice until analysis. The experiments included comparisons of cells labeled with various concentrations of the dyes and analyzed after storage for various times in order to determine the stability of these labeled cells. Cells were labeled with the membrane potential sensitive dyes $DiOC_6(3)$ or Di-4-ANEPPS by adding 5 μl/ml of the stock solution and incubating for 10-40 minutes at room temperature.

Stimulation of Cells

The induction of oxidative activity was detected with cells loaded with DCHF-DA or with CDDF-DA. These cells, suspended in BSS or in buffered saline sheath fluid, were incubated with 1.0 μl/ml of the stock solution of DCHF-DA or CDDF-DA for 20 minutes at room temperature. Aliquots (0.5 ml) of these loaded cells were then added to tubes containing 5 μl of DMSO, ethanol, or fMLP or PMA in these solvents, and incubated at 37° for 20 minutes. The activated cells were kept on ice until the flow cytometric analysis was completed. Changes in membrane potential were detected with $DiOC_6(3)$. Cells were incubated with 5 μl/ml of the 10 μM $DiOC_6(3)$ stock solution for 20-40 minutes at room temperature. The cells were then added to stimulators as described

above, incubated for 5 minutes at 37°, and kept on ice until flow cytometric analysis.

Flow Cytometry

Most flow cytometric analyses were performed with a Becton-Dickinson (Mountain View, CA 94043) FACS Analyzer connected to a Consort 30 (Hewlett-Packard 220) computer with a Consort 30 software package. The FACS Analyzer was equipped with a right angle light scatter detector and the standard FITC-PE optical filter set that produced incident light of 490 nm. Electronic (Coulter) volume (VOL), right angle light scatter (SSC), green (FL1, 520 nm) and red (FL2, ≥550 nm) fluorescence could be simultaneously detected for each cell. To measure Indo-1 fluorescence, a different set of optical filters producing 360 nm incident light and measuring 405 nm (FL1) and 485 nm (FL2) fluorescent light was used. Three decade logarithmic amplification was used with all parameters and digitized with an 8-bit analog to digital converter. With this arrangement, a change in signal intensity of 85 channels was equivalent to a ten-fold difference in intensity. The instrument was aligned and standardized with 7.8 μm fluorescein-conjugated beads having 4.3×10^5 fluoresceins/bead (Becton-Dickinson). In some experiments, either whole milk or cells prepared as described above were also analyzed using a Becton-Dickinson FACStar Plus cytometer. The FACStar Plus is a laser-based instrument with a different type of fluidic system that employs a jet-in-air stream instead of an enclosed system, as used in the FACS Analyzer. Instead of an electronic volume sensor, the FACStar Plus uses low angle forward light scatter to estimate volume.

Effects of Milk Collection and Storage Conditions on the Cells Recovered

Pre- and post-milking samples were collected from individual quarters before and after regular milking to determine if milk sampling procedures affected the types of cells recovered. Aliquots of these samples were centrifuged, the cells were labeled with CMFDA, and examined by flow cytometry. The pre-milking samples contained relatively more granulocytes, and the post-milking samples contained relatively more lymphocytes and monocyte-macrophage-type cells. Both types of milk samples contained a complete spectrum of the cells.

To determine the stability of cells in milk, milk samples were analyzed at various times from 30 minutes to overnight. Mastitic milk samples that were rich in granulocytes were selected for comparison, since granulocytes are particularly fragile. There was little storage of milk at room temperature (20-23°C) for 30 minutes to about 8 hours. After overnight storage, there was a loss of intact granulocytes and an increase in the number of disintegrating granulocytes detectable with CMFDA. Nevertheless, even after standing overnight, there were still readily detectable intact neutrophils in the mastitic milk.

Example 1: Cells Labeled with Esterase-Sensitive Dyes

As stated above, although cells in milk can be identified by labeling with CMFDA, this dy was unsuitable for automated identification of viable cells because of its relatively poor signal-to-noise ratio. Consequently, other esterified dyes were tested to determine if any of them produced superior signal-to-noise ratios.

The cells in milk and/or in peripheral blood were labeled with CMFDA and compared with other aliquots of the same cells labeled with CFDA, SFDA, BCECF-AM, or FDA. Two of these, namely, SFDA and CFDA, were found to be essentially useless under the conditions tested. SFDA was extremely inefficient in labeling cells and produced low levels of fluorescence in viable cells. CFDA labeled cells to a higher intensity but produced very poor discrimination between intact cells and damaged cells or platelets. BCECF-AM and FDA were much superior to CMFDA, since they labeled more intensely at lower concentrations. For example, it was necessary to add 1-10 μg CMFDA/ml in order to produce adequate fluorescence. However, BCECF-AM or FDA produced labeling of comparable or higher intensity when used at a concentration 1-2 orders of magnitude lower. Furthermore, BCECF-AM or FDA produced excellent discrimination between intact cells and disintegrating cells or platelets. Viable cells labeled with BCECF-AM or with FDA had fluorescence intensities at least 1000-fold higher than the natural autofluorescence and 100-fold higher than the signals produced by dead or damaged cells. These dyes can be used with other types of instruments to screen milk samples in order to detect those with elevated numbers of viable cells.

Indo-1 labeled cells were not directly compared with CMFDA-labeled cells because of the different fluorescent properties of the dyes. However, Indo-1 AM labeled viable blood cells much more intensely that dead or damaged cells. Thus, it appears that Indo-1 AM (Redelman D: 1988, Cytometry 9:156-163), could also be used if it is excited with the appropriate UV light.

The fluorescent dyes produced by intracellular de-esterification of non-fluorescent precursors are known to leak from cells. The more highly charged forms, such as the carboxyfluoresceins produced from CMFDA or CFDA, were

less susceptible to leakage. However, these dyes discriminate less effectively between viable and damaged cells and label viable cells less efficiently. Therefore, the stability of fluorescence in FDA-labeled cells was compared with CMFDA-labeled cells. Cells from bovine blood and/or milk were labeled with FDA or with CMFDA. These cells were analyzed immediately after labeling and after an additional four hours at ice temperature. There was a loss of 10-38% in fluorescence intensity after four hours at ice temperature, but essentially no difference was observed between the cells labeled with the two dyes. Thus, although fluorescein leaks more rapidly than carboxyfluorescein derivatives at higher temperatures, there is little difference in dye retention at ice temperature.

Example 2: Cells Labeled with Nucleic Acid Binding Dyes

Dihydroethidium is a unique dye that can be used to label selectively the nucleic acid of viable cells. When tested with cells from milk, it had at least three significant advantages in identifying and/or enumerating viable cells. First, HED labeled viable cells with a very high signal to noise ratio. The fluorescence intensity of viable cells after incubation with HED was as much as 10,000 fold higher than the intensity of autofluorescence. Second, the fluorochrome in viable cells is strongly bound. The reduced, non-charged HED is incorporated into viable cells where it is converted by intracellular oxidation to the charged ethidium ion. The intracellular ethidium binds to nucleic acid with very high affinity. Thus, viable cells are selectively labeled with a dye that binds tightly to intracellular materials. Third, the spectral properties of ethidium produced by intracellular oxidation of HED offers several useful features. The chief advantage is that ethidium has a very large Stokes shift making it relatively easy

to select optical filters that discriminate efficiently between the excitation and emission wavelengths. Therefore, HED can be used to identify viable cells either by flow cytometric techniques, as has been demonstrated, or by other procedures more suitable for processing large numbers of samples.

Example 3: Cells Labeled with Dyes Dependent Upon Intracellular Oxidation

Three dyes were examined that are converted from precursors to fluorescent forms via oxidation. Two of these dyes, i.e., DCHF-DA and CDDF-DA, require intracellular de-esterification prior to oxidation. The intracellular esterases operate at room temperature, as shown with other esterified dyes such as FDA, BCECF-AM, or CMFDA. On the other hand, the oxidative reaction requires higher temperatures for efficient conversion. Thus, neither the esterified dyes nor the third dye, HED, could be used effectively to label cells at room temperature.

To compare the effectiveness of these dyes in detecting granulocyte oxidative responses, cells from blood and/or milk were incubated at 37° for 15-20 minutes with the dyes alone or in the presence of various concentrations of PMA. The phorbol esters, such as PMA, are capable of activating granulocytes and causing an increase in the intracellular oxidative activity. Granulocytes from bovine or human blood that were labeled with DCHF-DA increased in fluorescence intensity by 10-30-fold when stimulated with PMA. The carboxy derivative, CDDF-DA, labeled granulocytes less efficiently and produced a relatively small increment of fluorescence when stimulated with PMA. Cells labeled effectively with HED as noted above, but produced a relatively modest increment in fluorescence when stimulated with PMA. DCHF-DA was found to be superior in

detecting oxidative responses in granulocytes. When labeled with DCHF-DA, the granulocytes in mastitic milk had fluorescence levels 3-10 fold higher than in granulocytes from blood. PMA stimulation caused an additional 3-8 fold increment of DCF fluorescence in granulocytes from milk. The results indicate that granulocytes in milk were preactivated in vivo but remained capable of producing an additional oxidative response. These results are consistent with the observation that granulocytes in the blood of burn patients are also preactivated. These results demonstrate that previous reports that granulocytes in milk are essentially unable to produce an oxidative response are not completely accurate. Since the cells are preactivated, the increment of response was decreased, but the cells retained the ability to respond to PMA.

Example 4: Cells Labeled with Membrane Potential Sensitive Dyes

Cells separated from milk by centrifugation and labeled with $DiOC_6(3)$ could be differentially identified by flow cytometry about as well as cells labeled with CMFDA. In addition, cells in whole milk could also be labeled with $DiOC_6(3)$ and identified using the FACStar flow cytometer. These dyes provided a poorer signal-to-noise ratio in attempts to discriminate between live and dead cells. However, dyes such as $DiOC_6(3)$ can also measure the degree of membrane depolarization, and hence the degree of activation of granulocytes.

The fluorescence intensity of cells labeled with $DiOC_6(3)$ is directly related to membrane potential. It was found that granulocytes and lymphocytes from bovine and human blood labeled with comparable intensity with $DiOC_6(3)$. The blood granulocytes from both species increased in fluorescence when hyperpolarized by exposure

to valinomycin. These cells decreased in fluorescence when treated with stimulatory amounts of PMA. However, the milk granulocytes neither hyperpolarized with valinomycin, nor did they change in intensity when exposed to PMA. The fluorescence intensity of the milk granulocytes was essentially the same as granulocytes from blood after stimulation with PMA. These results indicate that granulocytes from mastitic milk had been activated in vivo. These tests confirmed the results measuring intracellular oxidation, as described above.

The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

WHAT IS CLAIMED IS:

1. In a process for detecting, identifying, and/or enumerating viable cells which are present in a milk sample, wherein the viable cells while still in the milk or in another liquid phase, or after deposition on a solid phase, are fluorescently labeled with fluorescent dye and then identified and/or counted, the improvement wherein the viable cells are contacted, until the viable cells are fluorescently labeled, at a temperature at which cell membranes remain intact and at which appropriate cell enzymes are active, with a membrane permeant and non-fluorescent ester of a charged fluorescent dye, which ester can enter viable cells and can be converted by intracellular esterase enzymes to a charged fluorescent dye which produces fluorescence intensities in the viable cells which the ester enters which are at least ten fold greater than that which the ester produces in dead or damaged cells.

2. The process of claim 1, wherein the ester is added directly to milk.

3. The process of claim 1, wherein the cells are first separated from milk.

4. The process of claim 1, wherein the esterase dependent vital dye is fluorescein diacetate, 2',7'-biscarboxyethyl-5(6)-carboxyfluorescein acetoxymethyl ester, or Indo-1 acetoxymethyl ester.

5. The process of claim 1, wherein the milk sample is, or the cells separated therefrom are, incubated with the dye for a period of from about 5 to 30 minutes and at a temperature of from about 20° to 40° C.

6. The process of claim 1, wherein the resultant labeled cells from bovine milk are analyzed using flow cytometric techniques.

7. The process of claim 1, wherein the resultant labeled cells are separated or concentrated from the milk or liquid base and analyzed by exciting the fluorescent dye in the cells with a light source whose light comprises an exciting wavelength.

8. The process of claim 7, wherein the separation is effected by filtration.

9. The process of claim 7, wherein the separation is effected by centrifugation.

10. The process of claim 7, wherein the fluorescent light emitted from the labeled cells is detected by a photomultiplier tube or other light sensing device with sufficient sensitivity to detect and quantitate the emitted light.

11. In a process for selectively detecting, identifying, and/or enumerating viable cells in a milk sample, wherein viable cells still in the milk or in another liquid phase, or after deposition on a solid phase, are labeled with a fluorescent dye and then identified and/or counted, the improvement wherein the viable cells are contacted until the viable cells are fluorescently labeled, at a temperature at which cell membranes remain intact, appropriate cell enzymes are

active and the nucleic acid of the cells is intact, with a modified nucleic acid binding fluorescent dye that is chemically modified such that (a) its fluorescent properties are altered and/or its nucleic acid binding capacity is significantly diminished, (b) the chemically modified dye can enter viable cells, (c) intracellular enzyme(s) or other reactants can convert the chemically modified form to its fluorescent nucleic acid binding form, and (d) the nucleic acid of viable cells is, by the intracellularly converted form, fluorescently labeled with an intensity at least ten fold greater than in dead or damaged cells.

12. The process of claim 11, wherein the precursor of the nucleic acid binding vital dye is added directly to milk.

13. The process of claim 11, wherein the cells have been separated from milk.

14. The process of claim 11, wherein the dye is dihydroethidium.

15. The process of claim 11, wherein the milk sample or the cells separated therefrom are incubated with the chemically modified dye for a period of from about 5 to 30 minutes at a temperature from about 20° to 40°C.

16. The process of claim 11, wherein the resultant labeled cells from bovine milk are analyzed using flow cytometric techniques.

17. The process of claim 11, wherein the resultant labeled cells from bovine milk are separated or concentrated from the supernatant or from the milk and

analyzed by exciting the fluorescent dye in the cells with a light source whose light comprises an exciting wavelength.

18. The process of claim 17, wherein the separation is effected by filtration.

19. The process of claim 17, wherein the separation is effected by centrifugation.

20. The process of claim 17, wherein the fluorescent light emitted from the labeled cells is detected by a photomultiplier tube or other light sensing device with sufficient sensitivity to detect and quantitate the emitted light.

21. In a process for detecting, identifying, and/or enumerating viable cells which are present in a milk sample, wherein viable cells still in the milk, in another liquid phase, or after deposition on a solid phase, are labeled with a fluorescent dye and then identified and/or counted, the improvement wherein the viable cells are contacted until the viable cells are fluorescently labeled, at a temperature at which cell membranes are intact and at which appropriate cell enzymes are active, with a fluorescent dye modified by chemical reduction, or by reduction and additional chemical change such as esterification, such that (a) the fluorescence properties of the dye are altered, (b) the modified derivative can enter viable cells, and (c) the modified derivative can be converted by intracellular reactions to the parental fluorescent dye causing viable inflammatory cells to produce fluorescence intensities at least ten fold greater than that in dead or damaged cells and causing the ultimate intensity of fluorescence to be

directly related to the amount of intracellular oxidative activity.

22. The process of claim 21, wherein the precursor of the oxidation dependent vital dye is added directly to milk.

23. The process of claim 21, wherein the cells have been separated from the milk.

24. The process of claim 21, wherein the precursor dye is dihydrorhodamine 123.

25. The process of claim 21, wherein the precursor dye is the diacetate ester of dichlorodihydrofluorescein.

26. The process of claim 24, wherein the milk or the cells from the milk are incubated with the precursor dye for a period of from about 5 to 30 minutes at a temperature of from about 36° to 40° C.

27. The process of claim 25, wherein the milk or the cells from the milk are incubated with the precursor dye in a two step process with the initial incubation being at a temperature of 20° to 25° C for a period of from about 5 to 20 minutes and with the second incubation being at a temperature of from about 36° to 40° C for a period of from about 5 to 20 minutes.

28. The process of claim 21, wherein a portion of the milk containing the cells or a portion of a liquid vehicle containing the cells separated from the milk, or a portion of the cells after deposition on a solid phase, is stimulated with phorbol myristate acetate or other comparable agent which causes a maximal oxidative response in granulocytes.

29. The process of claim 21, wherein the resulting labeled cells are analyzed by flow cytometric techniques.

30. The process of claim 21, wherein the resultant labeled cells are separated or concentrated from the milk or other supernatant liquid and analyzed by exciting the fluorescent dye in the cells with light source whose light comprises an exciting wavelength.

31. The process of claim 30, wherein the separation is effected by filtration.

32. The process of claim 30, wherein the separation is effected by centrifugation.

33. The process of claim 30, wherein the fluorescent light emitted from the labeled cells is detected by a photomultiplier tube or other light sensing device with sufficient sensitivity to detect and quantitate the emitted light.

34. The process of claim 28, wherein the fluorescence produced by cells labeled without additional stimulation is compared with the fluorescence produced by cells maximally stimulated in order to estimate the content and/or activity of granulocytes in the milk.

35. The process of claim 1, wherein the process is used in the detection and treatment of mastitic cattle or in the determination of bovine milk quality.

36. The process of claim 11, wherein the process is used in the detection and treatment of mastitic cattle or in the determination of bovine milk quality.

37. The process of claim 21, wherein the process is used in the detection and treatment of mastitic cattle or in the determination of bovine milk quality.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/05377

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12Q 1/02
U.S. Cl: 435/29

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
U.S. Cl.	424/3; 435/4, 29

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

See attached

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	Cytometry, Volume 9, issued 1988, Reddman et al, "Identification of Inflammatory Cells in Bovine Milk by Flow Cytometry", pages 463-468, see entire document.	1-37
Y	Chemical Abstracts, vol. 81, No. 9, issued 1989, Ross et al., "Estimation of Cell Survival by Flow Cytometric Quantification of Fluorescein Diacetate Propidium Iodide Viable Cell Number", pages 3776-3782, see abstract No. 70228q, Cancer Research, 49(11), 1989.	1-37
Y	Chemical Abstracts, vol. 111, issued 1989, Jung et al, "Estimation of Matrix pH in Isolated Heart Mitochondria using a Fluorescent Probe", pages 348-351, see abstract No. 203356, Analy. Chem., 178(2), 1989.	1-37

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17 June 1991	28 OCT 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Jane Williams

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Documentation Searched

BIOSIS

MASTITIS
DIAGNOS?
FLOW CYTOMETRY
FLUORESCENT DYE
AU=REDELMAN, D

Chem Abstracts

ESTERASE DEPENDENT VITAL DYE
ESTERASE DEPENDENT
FLUORESCEIN DIACETATE
CARBOXYFLUORESCEIN ACETOXYMETHYL ESTER
ACETOXYMETHYL ESTER

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers___________, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.